

ESTERASES

RELATED APPLICATIONS

This application is a divisional of co-pending U. S. Patent Application Serial Number 08/602,359, filed February 17, 1996.

SPECIFICATION

This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production and isolation of such polynucleotides and polypeptides. More particularly, the polynucleotides and polypeptides of the present invention have been putatively identified as esterases. Esterases are enzymes that catalyze the hydrolysis of ester groups to organic acids and alcohols.

Many esterases are known and have been discovered in a broad variety of organisms, including bacteria, yeast and higher animals and plants. A principal example of esterases are the lipases, which are used in the hydrolysis of lipids, acidolysis(replacement of an esterified fatty acid with a free fatty acid) reactions, transesterification(exchange of fatty acids between triglycerides)reactions, and in ester synthesis. The major industrial applications for lipases include: the detergent industry, where they are employed to decompose fatty materials in laundry stains into easily removable hydrophilic substances; the food and beverage industry where they are used in the manufacture of cheese, the ripening and flavoring of cheese, as antistaling agents for bakery products, and in the production of margarine and other spreads with natural

butter flavors; in waste systems; and in the pharmaceutical industry where they are used as digestive aids.

The polynucleotides and polypeptides of the present invention have been identified as esterases as a result of their enzymatic activity.

In accordance with one aspect of the present invention, there are provided novel enzymes, as well as active fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding the enzymes of the present invention including mRNAs, cDNAs, genomic DNAs as well as active analogs and fragments of such enzymes.

In accordance with another aspect of the present invention there are provided isolated nucleic acid molecules encoding mature polypeptides expressed by the DNA contained in ATCC Deposit No. _____.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptides by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a nucleic acid sequence of the present invention, under conditions promoting expression of said enzymes and subsequent recovery of said enzymes.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes for hydrolyzing ester groups to yield an organic acid and an alcohol. The esterases of the invention are stable at high temperatures and in organic solvents and, thus, are superior for use in production of optically pure chiral compounds used in pharmaceutical, agricultural and other chemical industries.

In accordance with yet a further aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to hybridize to a nucleic acid sequence of the present invention.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such enzymes, or polynucleotides encoding such enzymes, for *in vitro* purposes related to scientific research, for example, to generate probes for identifying similar sequences which might encode similar enzymes from other organisms by using certain regions, *i.e.*, conserved sequence regions, of the nucleotide sequence.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims.

Figure 1 is an illustration of the full-length DNA (SEQ ID NO:23) and corresponding deduced amino acid sequence (SEQ ID NO:33) of *Staphylothermus marinus* F1-12LC of the present invention. Sequencing was performed using a 378 automated DNA sequencer (Applied Biosystems, Inc.) for all sequences of the present invention.

Figure 2 is an illustration of the full-length DNA (SEQ ID NO:24) and corresponding deduced amino acid sequence (SEQ ID NO:34) of *Pyrodictium* TAG11-17LC.

Figure 3 is an illustration of the full-length DNA (SEQ ID NO:25) and corresponding deduced amino acid sequence (SEQ ID NO:35) of *Archaeoglobus venificus* SNP6-24LC.

Figure 4 is an illustration of the full-length DNA (SEQ ID NO:26) and corresponding deduced amino acid sequence (SEQ ID NO:36) of *Aquifex pyrophilus*-28LC.

Figure 5 is an illustration of the full-length DNA (SEQ ID NO:27) and corresponding deduced amino acid sequence (SEQ ID NO:37) of M11TL-29L.

Figure 6 is an illustration of the full-length DNA (SEQ ID NO:28) and corresponding deduced amino acid sequence (SEQ ID NO:38) of *Thermococcus* CL-2-30LC.

Figure 7 is an illustration of the full-length DNA (SEQ ID NO:29) and corresponding deduced amino acid sequence (SEQ ID NO:39) of *Aquifex* VF5-34LC.

Figure 8 is an illustration of the full-length DNA (SEQ ID NO:30) and corresponding deduced amino acid sequence (SEQ ID NO:40) of *Teredinibacter*-42L.

Figure 9 is an illustration of the full-length DNA (SEQ ID NO:31) and corresponding deduced amino acid sequence (SEQ ID NO:41) of *Archaeoglobus fulgidus* VC16-16MC.

Figure 10 is an illustration of the full-length DNA (SEQ ID NO:32) and corresponding deduced amino acid sequence (SEQ ID NO:42) of *Sulfolobus solfataricus* P1-8LC.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequences ultimately process to produce the desired protein.

"Recombinant" enzymes refer to enzymes produced by recombinant DNA techniques; *i.e.*, produced from cells transformed by an exogenous DNA construct encoding the desired enzyme. "Synthetic" enzymes are those prepared by chemical synthesis.

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular enzyme, is a DNA sequence which is transcribed and translated into an enzyme when placed under the control of appropriate regulatory sequences.

In accordance with an aspect of the present invention, there are provided isolated nucleic acids (polynucleotides) which encode for the mature enzymes having the deduced amino acid sequences of Figures 1-10 (SEQ ID NOS:23-32).

In accordance with another aspect of the present invention, there are provided isolated polynucleotides encoding the enzymes of the present invention. The deposited material is a mixture of genomic clones comprising DNA encoding an enzyme of the present invention. Each genomic clone comprising the respective DNA has been inserted into a pBluescript vector (Stratagene, La Jolla, CA). The deposit has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA, on December 13, 1995 and assigned ATCC Deposit No. _____.

The deposit(s) have been made under the terms of the Budapest Treaty on the International Recognition of the deposit of micro-organisms for purposes of patent

procedure. The strains will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit would be required under 35 U.S.C. §112. The sequences of the polynucleotides contained in the deposited materials, as well as the amino acid sequences of the polypeptides encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The polynucleotides of this invention were originally recovered from genomic gene libraries derived from the following organisms:

Staphylothermus marinus F1 is a thermophilic sulfur archaea which was isolated in Vulcano, Italy. It grows optimally at 85°C ($T_{max} = 98°C$) at pH 6.5.

Pyrodictium TAG11 is a thermophilic sulfur archaea which was isolated in the Middle Atlantic Ridge. It grows optimally at 103°C ($T_{max} = 110°C$) at pH 6.5.

Archaeoglobus venificus SNP6 was isolated in the Middle Atlantic Ridge and grows optimally at 75°C ($T_{max} = 92°C$) at pH 6.9.

Aquifex pyrophilus K01 5a was isolated at Kolbeinsey Ridge, North of Iceland. This marine organism is a gram-negative, rod-shaped, strictly chemolithoautrophic, knall gas bacterium. It grows optimally at 85°C ($T_{max} = 95°C$) at pH 6.8.

M11TL is a new species of *Desulfurococcus* which was isolated from Diamond Pool (formerly Jim's Black Pool) in Yellowstone. The organism grows heterotrophically by fermentation of different organic materials (sulfur is not necessary) in grape-like aggregates optimally at 85 - 88°C in a low salt medium at pH 7.0.

Thermococcus CL-2 was isolated in the North Cleft Segment of the Juan de Fuca Ridge from a severed alvinellid worm residing on a "black smoker" sulfide structure. This marine archaea forms pleomorphic cocci, and grows optimally at 88°C.

Aquifex VF5 was isolated at a beach in Vulcano, Italy. This marine organism is a gram-negative, rod-shaped, strictly chemolithoautotrophic, knall gas bacterium. It grows optimally at 85°C ($T_{max} = 95^\circ\text{C}$) at pH 6.8.

Teredinibacter (pure) is an endosymbiont of the shipworm *Bankia gouldi*. The organism has straight to slightly bent 5-10 μm rods, and forms spiral cells as stationary phase is met. The organism was described in Science (1983) 22:1401-1403. It grows optimally at 30°C at pH 8.0.

Archaeoglobus fulgidus VC16 was isolated in Vulcano, Italy. The organism grows optimally at 85°C ($T_{max} = 92^\circ\text{C}$) at pH 7.0.

Sulfolobus solfataricus P1 grows optimally at 85°C ($T_{max} = 87^\circ\text{C}$) at pH 2.0.

Accordingly, the polynucleotides and enzymes encoded thereby are identified by the organism from which they were isolated, and are sometimes hereinafter referred to as F1/12LC (Figure 1 and SEQ ID NOS:23 and 33), TAG11/17LC (Figure 2 and SEQ ID NOS:24 and 34), SNP6/24LC (Figure 3 and SEQ ID NOS:25 and 35), AqP/28LC (Figure 4 and SEQ ID NOS:26 and 36), M11TL/29L (Figure 5 and SEQ ID NOS:27 and 37), CL-2/30LC (Figure 6 and SEQ ID NOS:28 and 38), VF5/34LC (Figure 7 and SEQ ID NOS:29 and 39), Trb/42L (Figure 8 and SEQ ID NOS:30 and 40), VC16/16MC (Figure 9 and SEQ ID NOS:31 and 41) and P1/8LC (Figure 10 and SEQ ID NOS: 32 and 42).

The polynucleotides and polypeptides of the present invention show identity at the nucleotide and protein level to known genes and proteins encoded thereby as shown in Table 1.

Table 1

Enzyme	Gene w/closest Homology (Organism)	Protein Similarity (%)	Protein Identity (%)	DNA Identity (%)
F1/12LC	No significant homology	-	-	-
TAG11/17LC	No significant homology	-	-	-
SNP6/24LC	PIR S34609 - carboxylesterase <i>Pseudomonas sp.</i> (strain KW1-56) open reading frame of unknown function in <i>E. coli</i> . <i>✓</i>	46	27	42
AqP 29 LC		53	31	38
M11TL/29LC	No significant homology	-	-	-
CL02/30LC	No significant homology	-	-	-
VF5/34LC	Identified by homology to 28LC; also homologous to ORF of unknown function 5' of tgs in <i>E. coli</i>	84	71	71
Trb/42L	No significant homology	-	-	-
P1-8LC				
VC16-16MC				

All the clones identified in Table 1 encode polypeptides which have esterase activity.

This invention, in addition to the isolated nucleic acid molecules encoding the enzymes of the present invention, also provides substantially similar sequences. Isolated

nucleic acid sequences are substantially similar if: (i) they are capable of hybridizing under conditions hereinafter described, to the polynucleotides of SEQ ID NOS:23-32; (ii) or they encode DNA sequences which are degenerate to the polynucleotides of SEQ ID NOS:23-32. Degenerate DNA sequences encode the amino acid sequences of SEQ ID NOS:33-42, but have variations in the nucleotide coding sequences. As used herein, substantially similar refers to the sequences having similar identity to the sequences of the instant invention. The nucleotide sequences that are substantially the same can be identified by hybridization or by sequence comparison. Enzyme sequences that are substantially the same can be identified by one or more of the following: proteolytic digestion, gel electrophoresis and/or microsequencing.

One means for isolating the nucleic acid molecules encoding the enzymes of the present invention is to probe a gene library with a natural or artificially designed probe using art recognized procedures (see, for example: Current Protocols in Molecular Biology, Ausubel F.M. *et al.* (EDS.) Green Publishing Company Assoc. and John Wiley Interscience, New York, 1989, 1992). It is appreciated by one skilled in the art that the polynucleotides of SEQ ID NOS:23-32, or fragments thereof (comprising at least 12 contiguous nucleotides), are particularly useful probes. Other particularly useful probes for this purpose are hybridizable fragments of the sequences of SEQ ID NOS:1-22 (*i.e.*, comprising at least 12 contiguous nucleotides).

With respect to nucleic acid sequences which hybridize to specific nucleic acid sequences disclosed herein, hybridization may be carried out under conditions of reduced stringency, medium stringency or even stringent conditions. As an example of oligonucleotide hybridization, a polymer membrane containing immobilized denatured nucleic acids is first prehybridized for 30 minutes at 45°C in a solution consisting of 0.9 M NaCl, 50 mM NaH₂PO₄, pH 7.0, 5.0 mM Na₂EDTA, 0.5% SDS, 10X Denhardt's, and 0.5 mg/mL polyriboadenylic acid. Approximately 2 X 10⁷ cpm (specific activity 4-9 X 10⁸ cpm/ug) of ³²P end-labeled oligonucleotide probe are then added to the solution. After 12-16 hours of incubation, the membrane is washed for 30 minutes at

room temperature in 1X SET (150 mM NaCl, 20 mM Tris hydrochloride, pH 7.8, 1 mM Na₂EDTA) containing 0.5% SDS, followed by a 30 minute wash in fresh 1X SET at T_m -10°C for the oligo-nucleotide probe. The membrane is then exposed to auto-radiographic film for detection of hybridization signals.

Stringent conditions means hybridization will occur only if there is at least 90% identity, preferably at least 95% identity and most preferably at least 97% identity between the sequences. *See J. Sambrook et al., Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory (1989)* which is hereby incorporated by reference in its entirety.

As used herein, a first DNA (RNA) sequence is at least 70% and preferably at least 80% identical to another DNA (RNA) sequence if there is at least 70% and preferably at least a 80% or 90% identity, respectively, between the bases of the first sequence and the bases of the another sequence, when properly aligned with each other, for example when aligned by BLASTN.

The present invention relates to polynucleotides which differ from the reference polynucleotide such that the changes are silent changes, for example the change do not alter the amino acid sequence encoded by the polynucleotide. The present invention also relates to nucleotide changes which result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference polynucleotide. In a preferred aspect of the invention these polypeptides retain the same biological action as the polypeptide encoded by the reference polynucleotide.

The polynucleotides of this invention were recovered from genomic gene libraries from the organisms listed in Table 1. Gene libraries were generated in the Lambda ZAP II cloning vector (Stratagene Cloning Systems). Mass excisions were performed on these libraries to generate libraries in the pBluescript phagemid. Libraries were

generated and excisions were performed according to the protocols/methods hereinafter described.

The polynucleotides of the present invention may be in the form of RNA or DNA which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequences which encodes the mature enzymes may be identical to the coding sequences shown in Figures 1-10 (SEQ ID NOS:23-32) or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature enzymes as the DNA of Figures 1-10 (SEQ ID NOS:23-32).

The polynucleotide which encodes for the mature enzyme of Figures 1-10 (SEQ ID NOS:33-42) may include, but is not limited to: only the coding sequence for the mature enzyme; the coding sequence for the mature enzyme and additional coding sequence such as a leader sequence or a proprotein sequence; the coding sequence for the mature enzyme (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature enzyme.

Thus, the term "polynucleotide encoding an enzyme (protein)" encompasses a polynucleotide which includes only coding sequence for the enzyme as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the enzymes having the deduced amino acid sequences of Figures 1-10 (SEQ ID NOS:33-42). The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature enzymes as shown in Figures 1-10 (SEQ ID NOS:23-32) as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of the enzymes of Figures 1-10 (SEQ ID NOS:23-32). Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotides may have a coding sequence which is a naturally occurring allelic variant of the coding sequences shown in Figures 1-10 (SEQ ID NOS:23-32). As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded enzyme.

Fragments of the full length gene of the present invention may be used as hybridization probes for a cDNA or a genomic library to isolate the full length DNA and to isolate other DNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 10, preferably at least 15, and even more preferably at least 30 bases and may contain, for example, at least 50 or more bases. The probe may also be used to identify a DNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of genomic DNA to determine which members of the library the probe hybridizes to.

It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. The probes are thus useful to isolate

complementary copies of DNA from other sources or to screen such sources for related sequences.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode enzymes which either retain substantially the same biological function or activity as the mature enzyme encoded by the DNA of Figures 1-10 (SEQ ID NOS:23-32).

Alternatively, the polynucleotide may have at least 15 bases, preferably at least 30 bases, and more preferably at least 50 bases which hybridize to any part of a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotides of SEQ ID NOS:23-32, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% identity and more preferably at least a 95% identity to a polynucleotide which encodes the enzymes of SEQ ID NOS:33-42 as well as fragments thereof, which fragments have at least 15 bases, preferably at least 30 bases and most preferably at least 50 bases, which fragments are at least 90% identical, preferably at least 95% identical and most preferably at least 97% identical under stringent conditions to any portion of a polynucleotide of the present invention.

The present invention further relates to enzymes which have the deduced amino acid sequences of Figures 1-10 (SEQ ID NOS:23-32) as well as fragments, analogs and derivatives of such enzyme.

The terms "fragment," "derivative" and "analog" when referring to the enzymes of Figures 1-10 (SEQ ID NOS:33-42) mean enzymes which retain essentially the same biological function or activity as such enzymes. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature enzyme.

The enzymes of the present invention may be a recombinant enzyme, a natural enzyme or a synthetic enzyme, preferably a recombinant enzyme.

The fragment, derivative or analog of the enzymes of Figures 1-10 (SEQ ID NOS:33-42) may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature enzyme is fused with another compound, such as a compound to increase the half-life of the enzyme (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature enzyme, such as a leader or secretory sequence or a sequence which is employed for purification of the mature enzyme or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The enzymes and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or enzyme present in a living animal is not isolated, but the same polynucleotide or enzyme, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or enzymes could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The enzymes of the present invention include the enzymes of SEQ ID NOS:33-42 (in particular the mature enzyme) as well as enzymes which have at least 70% similarity (preferably at least 70% identity) to the enzymes of SEQ ID NOS:33-42 and more preferably at least 90% similarity (more preferably at least 90% identity) to the enzymes of SEQ ID NOS:33-42 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the enzymes of SEQ ID NOS:33-42 and also include portions of such enzymes with such portion of the enzyme generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two enzymes is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one enzyme to the sequence of a second enzyme.

A variant, *i.e.* a "fragment", "analog" or "derivative" polypeptide, and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr,

exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Most highly preferred are variants which retain the same biological function and activity as the reference polypeptide from which it varies.

Fragments or portions of the enzymes of the present invention may be employed for producing the corresponding full-length enzyme by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length enzymes. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of enzymes of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, *etc.* The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing enzymes by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing an enzyme. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, *e.g.*,

derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: LTR or SV40 promoter, the *E. coli*. *lac* or *trp*, the phage lambda P_L promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E. coli*.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; fungal cells, such as

yeast; insect cells such as *Drosophila S2* and *Spodoptera Sf9*; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example; Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBluescript II KS, ptrc99a, pKK223-3, pDR540, pRIT2T (Pharmacia); Eukaryotic: pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, pSVL, SV40 (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda P_R, P_L and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell

can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, J., *Basic Methods in Molecular Biology*, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the enzymes of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the enzymes of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples include the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, *e.g.*, the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), α -factor, acid phosphatase, or heat shock proteins,

among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated enzyme. Optionally, the heterologous sequence can encode a fusion enzyme including an N-terminal identification peptide imparting desired characteristics, *e.g.*, stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell*, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The enzyme can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The enzymes of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant

production procedure, the enzymes of the present invention may be glycosylated or may be non-glycosylated. Enzymes of the invention may or may not also include an initial methionine amino acid residue.

Esterases are a group of key enzymes in the metabolism of fats and are found in all organisms from microbes to mammals. In the hydrolysis reaction, an ester group is hydrolysed to an organic acid and an alcohol.

Esterases enantiomerically differentiate dicarboxylic diesters and diacetates of diols. Using the approach disclosed in a commonly assigned, copending provisional application Serial No. 60/008,316, filed on December 7, 1995 and entitled "Combinatorial Enzyme Development," the disclosure of which is incorporated herein by reference in its entirety, one could convert the enantiospecificity of the esterase. Further, the thermostable esterases are believed to have superior stability at higher temperatures and in organic solvents. Thus, they are better suited for use in rigorous production processes which require robust catalysts.

There are a number of industrial and scientific applications for esterases, such as those of the present invention, including:

- 1) Esterases are useful in the dairy industry as ripening starters for cheeses, such as the Swiss-type cheeses;
- 2) Esterases are useful in the pulp and paper industry for lignin removal from cellulose pulps, for lignin solubilization by cleaving the ester linkages between aromatic acids and lignin and between lignin and hemicelluloses, and for disruption of cell wall structure when used in combination with xylanase and other xylan-degrading enzymes in biopulping and biobleaching of pulps;

3) Esterases are useful in the synthesis of carbohydrate derivatives, such as sugar derivatives;

4) Esterases are useful, when combined with xylanases and cellulases, in the conversion of lignocellulosic wastes to fermentable sugars for producing a variety of chemicals and fuels;

5) Esterases are useful as research reagents in studies on plant cell wall structure, particularly the nature of covalent bonds between lignin and carbohydrate polymers in the cell wall matrix;

6) Esterases are also useful as research reagents in studies on mechanisms related to disease resistance in plants and the process of organic matter decomposition; and

7) Esterases are useful in selection of plants bred for production of highly digestible animal feeds, particularly for ruminant animals.

Antibodies generated against the enzymes corresponding to a sequence of the present invention can be obtained by direct injection of the enzymes into an animal or by administering the enzymes to an animal, preferably a nonhuman. The antibody so obtained will then bind the enzymes itself. In this manner, even a sequence encoding only a fragment of the enzymes can be used to generate antibodies binding the whole native enzymes. Such antibodies can then be used to isolate the enzyme from cells expressing that enzyme.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, *Nature*, 256:495-497, 1975), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today*

4:72, 1983), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96, 1985).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic enzyme products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic enzyme products of this invention.

Antibodies generated against an enzyme of the present invention may be used in screening for similar enzymes from other organisms and samples. Such screening techniques are known in the art, for example, one such screening assay is described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (2d Ed.), Cold Spring Harbor Laboratory, Section 12.21-12.28 (1989) which is hereby incorporated by reference in its entirety.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1 μ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20 μ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel *et al.*, *Nucleic Acids Res.*, 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., *et al.*, *Id.*, p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (2d Ed.), Cold Spring Harbor Press (1989).

Example 1

Bacterial Expression and Purification of Esterases

DNA encoding the enzymes of the present invention, SEQ ID NOS:33 through 42, were initially amplified from a pBluescript vector containing the DNA by the PCR technique using the primers noted herein. The amplified sequences were then inserted into the respective PQE vector listed beneath the primer sequences, and the enzyme was expressed according to the protocols set forth herein. The 5' and 3' primer sequences for the respective genes are as follows:

Staphylothermus marinus F1-12LC

5' CCGAGAATTCTTAAAGAGGAGAAATTAAC TATGTCTTTAACAAAGCACTCT
3' CGGAAGATCTCTATCGTTAATGTATGATT T
vector: pQET

Pyrodictium TAG11-17LC

5' CCGAGAATTCTTAAAGAGGAGAAATTAAC TATGAAACTCTTGAGCCCA CA EcoRI
3' CGGAAGATCTCGCGTACACCATCAGCCA C BglIII
vector: pQET

Archaeoglobus venificus SNP6-24LC

5' CCGAGAATTCTTAAAGAGGAGAAATTAAC TATGCCATATGTTAGGAATG GT
3' CGGAGGTACCTTAGAACTGTGCTGAAGAAA TAAATTCGTC CATTGCTCT
3' CGGAGGTACCTTAGAACTGTGCTGAAGAAA TAAATTCGTC CATTGCTCTA TTA
vector: pQET

Aquifex pyrophilus - 28LC

5' CCGAGAATTCTTAAAGAGGAGAAATTAAC TATGAGATTGAGGAAATTTG AAG
3' CGGAGGTACCTATTCAAGAAAGTACCTCTA A
vector: pQET

M11TL - 29LC

5' CCGAGAATTCTTAAAGAGGAGAAATTAAC TATGTTAATATCAATGTCTTT
3' CGGAAGATCTTTAAGGATTTTCCCTGGGTA G

vector: pQET

Thermococcus CL-2 - 30LC

5' CCGAGAATTTC ATTAAAGAGG AGAAATTAAC TATGGAGGTT TACAAGGCCA AA
3' CGGAGGTACC TTATTGAGCC GAAGAGTACG A

vector: pQET

Aquifex VF5 - 34LC

5' CCGAGAATTTC ATTAAAGAGG AGAAATTAAC TATGATTGGC AATTTGAAAT TGA
3' CGGAGGTACC TTAAAGTGCT CTCATATCCC C

EcoRI
KpnI

vector: pQET

Teredinibacter 42L

5' CCGAGAATTTC ATTAAAGAGG AGAAATTAAC TATGCCAGCT AATGACTCAC CC
3' CGGAAGATCT TCAACAGGCT CCAAATAATT TC (without His-tag)
3' CGGAAGATCT ACAGGCTCCA AATAATTTC (with His-tag)

vector: pQE12

Archaeoglobus fulgidus VC16-16MC

5' CCGAGAATTTC ATTAAAGAGG AGAAATTAAC TATGCTTGAT ATGCCAATCG AC
3' CGGAGGTACC CTAGTCGAAG ACAAGAAGAG C

EcoRI
KpnI

vector: pQET

Sulfolabus solfataricus P1-8LC

5' CCGAGAATTTC ATTAAAGAGG AGAAATTAAC TATGCCCCAG GATCCTAGAA TT
3' CGGAGGTACC TTAAATTTA TCATAAAATA C

EcoRI
KpnI

vector: pQET

The restriction enzyme sites indicated correspond to the restriction enzyme sites on the bacterial expression vector indicated for the respective gene (Qiagen, Inc. Chatsworth, CA). The pQE vector encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites.

The pQE vector was digested with the restriction enzymes indicated. The amplified sequences were ligated into the respective pQE vector and inserted in frame with the sequence encoding for the RBS. The ligation mixture was then used to transform the *E. coli* strain M15/pREP4 (Qiagen, Inc.) by electroporation. M15/pREP4 contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance

(Kan^r). Transformants were identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D. ⁶⁰⁰) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours. Cells were then harvested by centrifugation.

The primer sequences set out above may also be employed to isolate the target gene from the deposited material by hybridization techniques described above.

Example 2

Isolation of a Selected Clone from the Deposited Genomic Clones

The two oligonucleotide primers corresponding to the gene of interest are used to amplify the gene from the deposited material. A polymerase chain reaction is carried out in 25 μ l of reaction mixture with 0.1 μ g of the DNA of the gene of interest. The reaction mixture is 1.5-5 mM MgCl₂, 0.01% (w/v) gelatin, 20 μ M each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 1.25 Unit of Taq polymerase. Thirty cycles of PCR (denaturation at 94°C for 1 min; annealing at 55°C for 1 min; elongation at 72°C for 1 min) are performed with the Perkin-Elmer Cetus 9600 thermal cycler. The amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the gene of interest by subcloning and sequencing the DNA product.

Example 3

Production of the Expression Gene Bank

Colonies containing pBluescript plasmids with random inserts from the organisms M11TL, *Thermococcus GU5L5*, and *Teredinibacter* were obtained according to the method of Hay and Short, *Strategies*, 5:16, 1992.

Example 4

Screening for Lipase/Esterase Activity

The resulting colonies were picked with sterile toothpicks and used to singly inoculate each of the wells of 96-well microtiter plates. The wells contained 250 μ L of LB media with 100 μ g/mL ampicillin, 80 μ g/mL methicillin, and 10% v/v glycerol (LB Amp/Meth, glycerol). The cells were grown overnight at 37°C without shaking. This constituted generation of the "Source GeneBank." Each well of the Source GeneBank thus contained a stock culture of *E. coli* cells, each of which contained a pBluescript with a unique DNA insert.

The plates of the Source GeneBank were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200 μ L of LB Amp/Meth, glycerol. This step was performed using the High Density Replicating Tool (HDRT) of the Beckman Biomek with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each well of the Condensed Plate thus contained 10 to 12 different pBluescript clones from each of the source library plates. The Condensed Plate was grown for 16 hours at 37°C and then used to inoculate two white 96-well Polyfiltronics microtiter daughter plates containing in each well 250 μ L of LB Amp/Meth (no glycerol). The original condensed plate was put in storage -80°C. The two condensed daughter plates were incubated at 37°C for 18 hours.

The short chain esterase '600 μ M substrate stock solution' was prepared as follows: 25 mg of each of the following compounds was dissolved in the appropriate volume of

DMSO to yield a 25.2 mM solution. The compounds used were 4-methylumbelliferyl propionate, 4-methylumbelliferyl butyrate, and 4-methylumbelliferyl heptanoate. Two hundred fifty microliters of each DMSO solution was added to ca 9 mL of 50 mM, pH 7.5 Hepes buffer which contained 0.6% of Triton X-100 and 0.6 mg per mL of dodecyl maltoside (Anatrace). The volume was taken to 10.5 mL with the above Hepes buffer to yield a slightly cloudy suspension.

The long chain '600 μ M substrate stock solution' was prepared as follows: 25 mg of each of the following compounds was dissolved in DMSO to 25.2 mM as above. The compounds used were 4-methylumbelliferyl elaidate, 4-methylumbelliferyl palmitate, 4-methylumbelliferyl oleate, and 4-methylumbelliferyl stearate. All required brief warming in a 70°C bath to achieve dissolution. Two hundred fifty microliters of each DMSO solution was added to the Hepes buffer and diluted to 10.5 mL as above. All seven umbelliferones were obtained from Sigma Chemical Co.

Fifty μ L of the long chain esterase or short chain esterase '600 μ M substrate stock solution' was added to each of the wells of a white condensed plate using the Biomek to yield a final concentration of substrate of about 100 μ M.. The fluorescence values were recorded (excitation = 326 nm, emission = 450 nm) on a plate-reading fluorometer immediately after addition of the substrate. The plate was incubated at 70°C for 60 minutes in the case of the long chain substrates, and 30 minutes at RT in the case of the short chain substrates. The fluorescence values were recorded again. The initial and final fluorescence values were compared to determine if an active clone was present.

Example 5

Isolation and Purification of the Active Clone

To isolate the individual clone which carried the activity, the Source GeneBank plates were thawed and the individual wells used to singly inoculate a new plate containing LB Amp/Meth. As above, the plate was incubated at 37°C to grow the cells, 50 μ L of

600 μ M substrate stock solution was added using the Biomek and the fluorescence was determined. Once the active well from the source plate was identified, cells from this active well were streaked on agar with LB/Amp/Meth and grown overnight at 37°C to obtain single colonies. Eight single colonies were picked with a sterile toothpick and used to singly inoculate the wells of a 96-well microtiter plate. The wells contained 250 μ L of LB Amp/Meth. The cells were grown overnight at 37°C without shaking. A 200 μ L aliquot was removed from each well and assayed with the appropriate long or short chain substrates as above. The most active clone was identified and the remaining 50 μ L of culture was used to streak an agar plate with LB/Amp/Meth. Eight single colonies were picked, grown and assayed as above. The most active clone was used to inoculate 3 mL cultures of LB/Amp/Meth, which were grown overnight. The plasmid DNA was isolated from the cultures and utilized for sequencing.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.